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# Fractionation of Gelatins using Amberlite CG-50 Resin

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By means of an Amberlite CG-50 resin column, it is possible to separate gelatins into a continuous series of arbitrary fractions by eluting with buffers of increasing pH. The distribution pattern of acid (lime-treated) gelatins was found to occur at a lower pH range than that of neutral gelatins. The fractions which emerged first had a slightly lower molecular weight.

It is suggested that the more acidic and more degraded fractions arise from the surfaces of the fibres.

This work was inspired by the publication by Russell [1] who succeeded in isolating, with a cation exchange resin, a "pH 5.5 fraction" (which passed through the Amberlite cation exchange resin at this pH) from lime-treated gelatin and claimed that it differed from the bulk material by its amino acid composition. We have observed that, with 0.1 N sodium hydroxide, the bulk of the gelatinized collagen from bone can be further fractionated [2]. In the present investigation, we wished to pay special attention to the bulk fractions. The work of Russell was extended by Leach [3] who concluded that the amount of the "pH 5.5 fraction" obtained depended partly on the conditions (pH, salt strength, amount of the sample and surface area of the resin) and partly on the properties of the gelatin (acidity and molecular weight). He did not find any marked differences between the "pH 5.5 fraction" and the bulk material.

Partridge and Boardman [4] have pointed out that the ionic strength is very important in the fractionation of proteins with resins. They stressed the use of higher temperatures which, with most proteins, involves a danger of denaturation but is an advantage with gelatins.

## EXPERIMENTAL

A 1 cm diameter, water-jacketed column was filled with a suspension (in the first buffer) of the resin which was allowed to settle to 15 cm height. (Between runs the resin was taken out, regenerated, washed and finally suspended again in the first buffer.) The fraction collector was steered by a timer to accept 2 ml fractions, which took 1 ½–3 min. The speed of elution was about 5–15 drops per min. This speed could be decreased to one-fourth without any appreciable effect on the fractionation pattern. If the run was interrupted, the next immediate fractions contained erroneously high amounts of protein and several artefact peaks were encountered in this way. The column was kept at a temperature of +36°C (pH 5.5) or +37°C (gradient and NaOH) but check experiments were performed at lower temperatures.

In the initial experiments, two eluents were used: (1) pH 5.5 citric acid-disodium phosphate buffer [5] (McIlwaine) and (2) 0.1 N sodium hydroxide. In the final procedure, the 0.1 N sodium hydroxide was replaced by various gradients of increasing salt strength and/or pH (see the figure legends). Some experiments were made using initially a pH 5.0 buffer.

The sample (usually 50 mg of gelatin, as 1 % solution) was heated to about + 60°C and allowed to cool down to + 37°C before application to the column.

The protein in the fractions was estimated colorimetrically in 0.25—0.5 ml aliquots using a modified biuret reagent [6]. It is known that this reagent does not give equal colours with different fractions but, for the location of the fractions, it is quite satisfactory.

Nine gelatins were studied:

I. commercial, turbid (cleared upon heating), formed poor jelly, pI 5.3;

II. commercial, clear, formed strong jelly, prepared from "cartilage and good bones", pI 5.2;

III. laboratory preparation according to Jackson [7], formed good jelly, turbid even when heated, pI 6.9;

IV. commercial, "for bacteriological purposes", clear, formed strong jelly, pI 7.5;

V. sample specially prepared by Kind & Knox Gelatin Company from porkskin (acid pretreatment), pI 6.8, Bloom value 304;

VI. second run after preparation V, pI 6.8, Bloom value 172;

VII. sample specially prepared by Kind & Knox Gelatin Company from limed calfskins, pI 4.95, Bloom value 244;

VIII. second run after preparation VII, pI 4.9, Bloom value 218;

IX. third run after preparations VI and VII, pI 4.85, Bloom value 124. Fractionation patterns of this sample resembled those of sample VIII and are not given.

The pI was determined as the pH of the gelatin solution which had passed through a mixed-bed resin to remove the ions [8].

The cation exchange resin (Amberlite CG-50, Type 1, 100—200 mesh, Lot 27743) was purchased from British Drug House Ltd., Poole, England.

When the pH of the fractions was raised to 7, a precipitate appeared in most of the fractions, especially when concentrated by evaporation. The physical examinations (diffusion and sedimentation) were therefore performed in an acetate buffer at pH 4.8, + 38°C and ionic strength of 0.15. The sedimentation was studied by means of a Spinco model E ultracentrifuge, and diffusion properties by Antweiler's microelectrophoresis apparatus using the diffusion cell. The diffusion was recorded by means of a built-in interferometric optical system.

## RESULTS

Fig. 1. gives the fractionation patterns of samples of different gelatins using the original method of Russell. The "pH 5.5 peak" from the more acid gelatins (I, II) is larger and the fraction eluted with 0.1 N sodium hydroxide is skewed to the left. To find out whether the "pH 5.5 peak" was an independent peak and not an "overflow" fraction, it was rechromatographed twice (Fig. 2) without concentration or any other manipulation. It seems evident that, on rechromatography, only an insignificant amount is retained in the resin. Thus the "pH 5.5 peak" represents a real fraction, but not a homogeneous one as shown below.

To elucidate whether the fractionation depended primarily on the pH of the eluent or on its salt strength, the NaOH elution was replaced by a concentration gradient (by increasing the concentration of McIlwaine's buffer at pH 5.5 threefold) or by a pH gradient (by increasing the pH gradually by means of a decrease in the citric acid in the buffer). No definite fraction emerged as a result of the increase in salt strength but, when the pH was raised, a distinct fraction was obtained.

The result was improved (Fig. 3) when the gradient was made steeper by the use of a more concentrated disodium phosphate solution in the reservoir

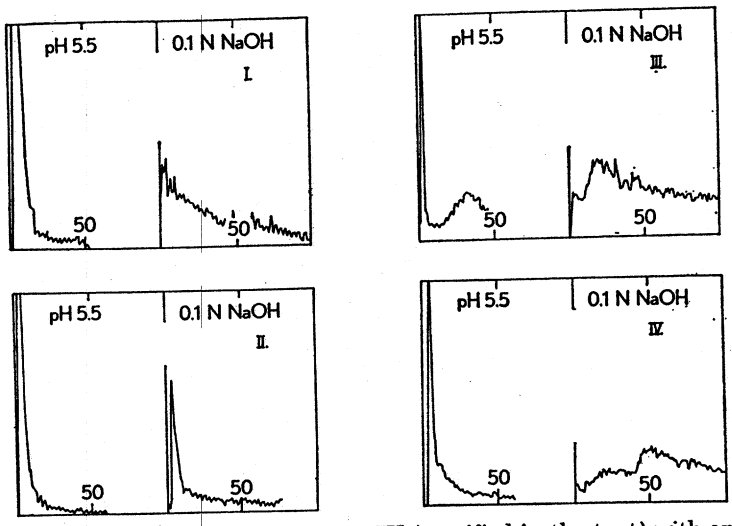


*Fig. 1.* Fractionation of various gelatins I—IV (specified in the text) with an Amberlite CG-50 resin column which was eluted first with pH 5.5 buffer and then with 0.1 N sodium hydroxide. Number of the fractions is given.

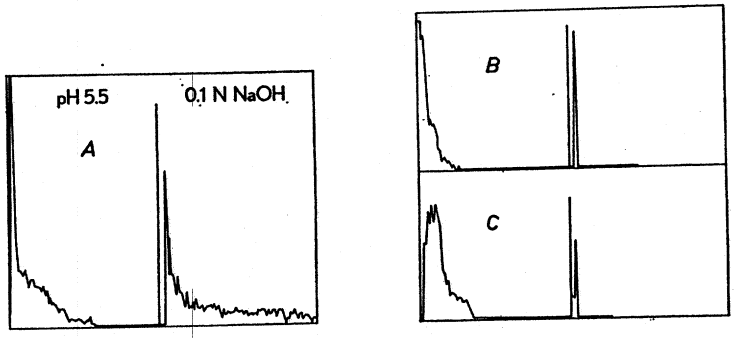


*Fig. 2.* Rechromatography of the "pH 5.5 fraction" obtained from gelatin II (Fig. 1.); A = original; B = "pH 5.5 fraction" of A, rechromatographed; C = "pH 5.5 fraction" of B, rechromatographed.

vessel. In "neutral" gelatins (III, IV, V, VI), the $Na_2HPO_4$-gradient fraction is much more marked than in "acid" gelatins (I, II, VII, VIII). From some gelatins (especially IV) a "residual" fraction was recovered using sodium hydroxide. Some additional experiments were made to find out whether the "pH 5.5 peak" could be divided into subfractions. Fig. 4 shows the "pH 5.5 peak" (from gelatin I) rechromatographed in this manner and it is obviously heterogeneous. Using a pH 5.0 citric acid-disodium phosphate buffer instead of the pH 5.5 buffer, a part of the gelatin was still found to pass through the column. When the pH 5.5 buffer was replaced by the pH 5.0 buffer, the bulk of the material emerged as a single fraction which obviously contained some of the material in the "pH 5.5 fraction" together with the "gradient-fraction"

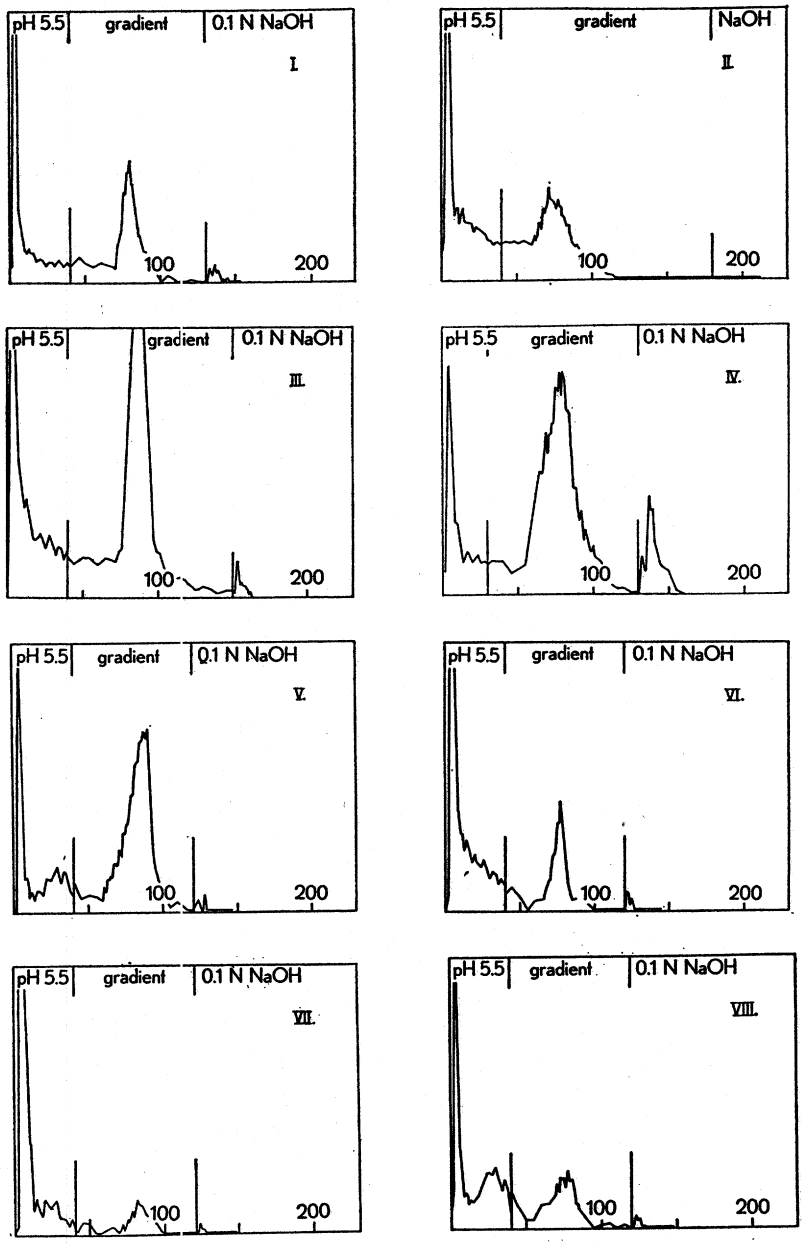


*Fig. 3.* Chromatography of various gelatins I—VIII (specified in the text) using a pH gradient from pH 5.5 upwards. The volume of the mixing vessel (from which the column was fed) was 100 ml and contained originally McIlwaine's buffer. This was replaced by 0.5 M $Na_2HPO_4$ from the reservoir vessel. The number of the 2 ml fractions is indicated.

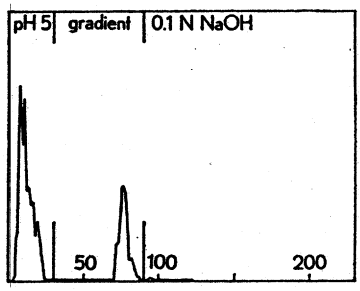


*Fig. 4.* Rechromatography of the "pH 5.5 fraction" of gelatin I using gradient (as in Fig. 3) beginning at a pH 5.0 (McIlwaine) buffer.

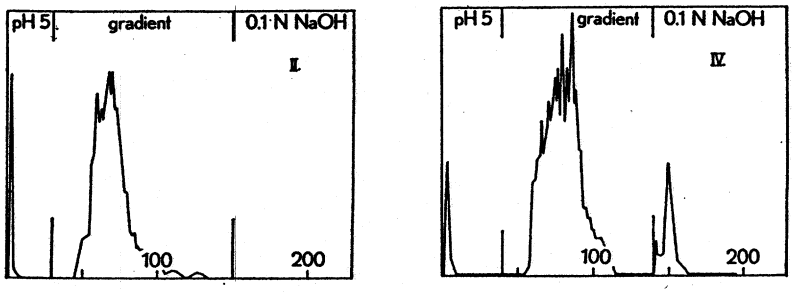


*Fig. 5.* Chromatography of total gelatins II and IV in the same condition as in Fig. 4.

(Fig. 5). There exists a continuous distribution of fractions which can be separated using buffers of suitable pH. It is not yet quite certain whether there is more than one peak in this distribution and also whether the fractions differ by other properties than their electrical charge. Several unsuccessful attempts were made to modify the gradient in order to split the bulk material which emerged within a range of about one pH unit. The "sodium hydroxide fraction" (*e.g.* from gelatin IV) seems to be different from the bulk fraction.

The sedimentation coefficient ($s^{\circ}_{20,w}$) of gelatin II was for the "pH 5.5 fraction" 2.64 S and for 0.1 N sodium hydroxide fraction 2.43 S. The diffusion coefficient of the "pH 5.5 fraction" was dependent on the time and $D^{\circ}_{20,w}$ was estimated to 4.4-5.7 $\times$ $10^{-7}$ cm$^2$/sec. For the NaOH-eluted fraction (Fig. 1, II) the diffusion coefficient was 3.0 $\times$ $10^{-7}$ cm$^2$/sec. Assuming $\overline{V} = 0.70$, the molecular weight of the "pH 5.5 fraction" was therefore about 40 000—50 000 and of the 0.1 N sodium hydroxide-eluted fraction about 65 000.

## DISCUSSION

There is ample evidence for the existence of two fractions in gelatins prepared by mild degradations of soluble collagens [9]. They are known to differ with respect to molecular weight and amino acid composition, *e.g.* the content of histidine. Further studies will show whether the fractions of smaller

particle weight, described above, differ in regard to amino acid composition. In this respect, the "sodium hydroxide fraction" (Fig. 3, IV) is of interest since, judging by its pI, it resembles closest the parent collagen [10,11].

It is not clear to us what is the basic reason for the fractionation. The first fraction emerging is of lower molecular weight. Also, the more acid gelatins contained larger non-adsorbed fractions. In these respects we can confirm the work of Leach [3]. The most plausible, but not necessarily the only reason for the fractionation, seems to us to be the difference in electrical charge. However, attempts to fractionate gelatins by electrophoresis on "Oxoid" cellulose acetate strips at + 37°C and pH 8.6 were not successful.

The technical properties, (jelly strength, turbidity) do not seem to be directly correlated with the fractionation pattern. When the fractionation was performed at + 4°C or in the presence of 4 M urea, that portion of gelatin (II) which was not retained by the column was decreased.

We are of the opinion that these fractions are formed by deamidation (and partly by modification of the arginine residue) during the soaking of the original collagenous material in lime or acid [12]. Some fractions of the collagen may be more exposed to deamidation. If all the amido groups of the collagen were exposed to deamidation at random, we would expect to get whole molecules (or their large parts) of similar acidity. It remains to be proved whether the more acidic fractions arise from the most recent deposits of collagen on or near the surface of the fibres. This suggestion is supported by the fact that soluble collagens yielded in preliminary experiments relatively large "pH 5.5 fractions", presumably because all the molecules were exposed to the degradation simultaneously.

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